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Chordin, FGF signaling, and mesodermal factors cooperate in zebrafish neural induction

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Abstract

The ectoderm gives rise to both neural tissue and epidermis. In vertebrates, specification of the neural plate requires repression of bone morphogenetic protein (BMP) signaling in the dorsal ectoderm. The extracellular BMP antagonist Chordin and other signals from the dorsal mesoderm play important roles in this process. We utilized zebrafish mutant combinations that disrupt Chordin and mesoderm formation to reveal additional signals that contribute to the establishment of the neural domain. We demonstrate that fibroblast growth factor (FGF) signaling accounts for the additional activity in neural specification. Impeding FGF signaling results in a shift of ectodermal markers from neural to epidermal. However, following inhibition of FGF signaling, expression of anterior neural markers recovers in a Nodal-dependent fashion. Simultaneously blocking, Chordin, mesoderm formation, and FGF signaling eliminates neural marker expression during gastrula stages. We observed that FGF signaling is required for *chordin* expression but that it also acts via other mechanisms to repress BMP transcription during late blastula stages. Activation of FGF signaling was also able to repress BMP transcription in the absence of protein synthesis. Our results support a model in which specification of anterior neural tissue requires early FGF-mediated repression of BMP transcript levels and later activities of Chordin and mesodermal factors.

Keywords: Neural induction; FGF; Nodal; Chordin; BMP; Zebrafish

Introduction

During embryogenesis, the neural plate, which will give rise to the entire central nervous system encompassing the brain and spinal cord, is specified within the ectoderm. The longstanding model for neural induction and initial polarization of the neural plate is the activator-transformer model first proposed by Nieukoop (1952). In this model, an activator signal distinguishes the neural ectoderm from nonneural ectoderm (epidermis). Initially, the neural ectoderm is anterior in character and is subsequently patterned by a transformation step to generate posterior fates. This model predicts that neural and nonneural ectoderms are in equilibrium; therefore, promotion of neural fates should come at the expense of epidermal fates. Conversely, impeding neural induction should expand the epidermal domain.

In classic experiments, Spemann (1924) and Mangold observed that transplantation of the dorsal mesoderm (termed the organizer) to a ventral location induced a complete secondary axis including a well-patterned neural tube. Elegant experiments in *Xenopus laevis* identified molecules expressed in the dorsal mesoderm that have potent neural inductive activity. Prominent among these molecules are *chordin* and *noggin* (Sasai et al., 1995; Smith and Harland, 1992). In vertebrates, neural induction occurs in a dorsal sector of the embryo where bone morphogenetic protein (BMP) signaling has been repressed (Hemmati-Brivanlou and Melton, 1997). Chordin and Noggin inhibit BMP signaling by binding extracellular BMP ligands and

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interfering with receptor activation (Piccolo et al., 1996; Zimmerman et al., 1996).

The activities of the organizer and extracellular BMP antagonists are not entirely linked. In zebrafish, dorsal cells with neural inductive activity reside outside the morphological boundaries of the organizer (Grinblat et al., 1998; Saude et al., 2000) and *chordin* expression also stretches beyond the organizer (Miller-Bertoglio et al., 1997). In the chick, the node is able to act as a neural inducer prior to the expression of known BMP antagonists (Streit et al., 1998). While experiments to knockdown Chordin function in *Xenopus* also support the notion that Chordin is an essential component of the neuralizing activity of the organizer (Oelgeschlager et al., 2003), recent experiments also suggest that Chordin is required outside of the organizer for specification of some anterior neural fates (Kuroda et al., 2004).

Despite the demonstration that both the organizer and the extracellular BMP antagonists are sufficient to induce neural tissue in a variety of assays, genetic evidence suggests that neural induction occurs in the absence of the organizer or extracellular BMP antagonists. Mouse and zebrafish mutants that lack the organizer still undergo neuralization (Ang and Rossant, 1994; Feldman et al., 1998; Gritsman et al., 1999; Klingensmith et al., 1999). Zebrafish mutants lacking the organizer still maintain dorsal expression of *chordin* (Gritsman et al., 1999; Sirotkin et al., 2000). In these mutants, specification of neural tissue may result from extracellular antagonism of BMP signaling by Chordin. The Chordin locus is disrupted in zebrafish *dino* mutants that have reductions in anterior neural tissues. Likewise, mice that are double mutant for *chordin* and *noggin* have anterior neural truncations (Bachiller et al., 2000; Schulte-Merker et al., 1997). Analysis of these mutants suggests that antagonism of BMP signaling by Chordin and related molecules is one key mechanism of neural induction but that additional signaling events play important roles.

One additional class of molecules that has been implicated in neural induction is the fibroblast growth factors (FGFs). Their role in neural induction has been controversial, and experiments in different model systems have indicated that there may be species-specific mechanisms of neural induction. Manipulations of chick embryos suggest a role for FGF in neural induction (Streit et al., 2000; Wilson et al., 2000, 2001) and that FGFs may attenuate BMP signaling by repressing the transcription of BMP4 and BMP7 (Wilson et al., 2000).

However, overexpression of a dominant-negative FGF receptor1 (XFD) or an inhibitory *ras* construct in zebrafish and frog embryos does not prevent formation of anterior neural structures (Amaya et al., 1991; Griffin et al., 1995; Ribisi et al., 2000). These embryos lack all posterior tissue, including spinal cord, but contain hindbrain and anterior neural structures. Furthermore, isolated XFD-expressing cells are capable of becoming spinal cord (Kroll and Amaya, 1996; Ribisi et al., 2000). Together, these results suggest that FGF signaling is not required for neural induction. However, Hongo et al. (1999) demonstrated that in an in vitro culture system blocking FGF signaling with Δ-FGFR-4 or to a lesser extent XFD inhibits neural induction by the organizer and blocks autonomous neuralization of cultured disassociated ectodermal cells. In those experiments, whole embryos injected with Δ-FGFR-4 mRNA still generated anterior neural tissue at late stages. One mechanism by which FGF may act as a neural inducer was suggested by the observation that BMP signaling can be quashed by FGF-mediated phosphorylation of the Smad1 linker region at consensus MAP–ERK kinase phosphorylation sites (Pera et al., 2003).

In this study, we show that in zebrafish, neural tissue is induced as a result of the combined activities of FGF signaling, Chordin and Nodal downstream targets. Inhibition of FGF signaling in wild-type embryos results in early deficits in neural specification and expansion of nonneural ectoderm. However, the anterior neural domain later recovers in a Nodal-dependent fashion following FGF inhibition. Our results demonstrate that FGF acts to diminish BMP transcript levels prior to the start of gastrulation. We show that while FGF induces expression of *chordin* transcripts, it also represses BMP transcript levels by a translation-independent mechanism. Together, these findings suggest that FGF acts at multiple levels to repress BMP signaling and define the neural territory.

Materials and methods

Zebrafish stocks and embryo maintenance

Adult zebrafish stocks were maintained at 28.5°C. Embryos were produced by natural matings of appropriate adult fish, collected and stored at 28.5°C in embryo medium until desired stage according to Kimmel et al. (1995). The following mutant alleles were used in this study: *dino*^{tt250}, *ntl*^{b160}, Mz*oep*^{tz57}, as well as TL wild-type fish.

Pharmacologic treatments

FGF signaling was pharmacologically inhibited by placing whole embryos of the appropriate stage into 60 μM SU5402 (Calbiochem, La Jolla, CA.). The inducible FGF receptor 1 (iFGFR-1) construct was activated at the appropriate stage with 1.25 μM AP20187 (ARIAD Pharmaceuticals, www.ariad.com/regulationkits). Embryos were left in AP20187 and allowed to develop to the appropriate stage. The iFGFR-1 construct did not have an effect unless embryos were placed into AP20187. Protein synthesis was inhibited by placing embryos into 1 μM cycloheximide (Sigma-Aldrich, St. Louis, MO) for 1 h.

mRNA synthesis and microinjections of mRNAs and morpholinos

Sense mRNA was made using the mMESSAGE mMACHINE RNA synthesis kit (Ambion). The XFD, Δ-FGFR-4, *fgf3*, and *chordin* (Amaya et al., 1991; Hongo et al., 1999; Kiefer et al., 1996; Miller-Bertoglio et al., 1997) constructs have been previously described. *ntl* morpholinos were synthesized by Gene Tools (Philomath, OR) and have been previously described (Nasevicius and Ekker, 2000). Prior to microinjections, embryos were dechorionated in 2 mg/ml pronase (Sigma-Aldrich). One- to four-cell embryos were injected with 0.5 nl RNA diluted in 0.2 M KCl and phenol red. At the appropriate stage, embryos were fixed in 4% paraformaldehyde for in situ hybridization or placed in TRIzol reagent (Invitrogen) for RNA extraction.

Whole mount in situ hybridization, photography, and genotyping

Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C then stored in 100% methanol for storage at −20°C. In situ hybridizations were done as previously described (Thisse et al., 1993). Constructs used to synthesize the following probes have been described previously: *bmp2*, *bmp4*, *cyp26*, *emx1*, *gata2*, *gata3*, *hoxb1b*, *krox20*, *otx2*, *opl*, *pax2.1*, *papc*, *spt*, and *tbx6* (Detrich et al., 1995; Griffin et al., 1998; Grinblat and Sive, 2001; Hug et al., 1997; Krauss et al., 1991; Kudoh et al., 2002; Mori et al., 1994; Morita et al., 1995; Neave et al., 1995; Nikaido et al., 1997; Oxtoby and Jowett, 1993; Yamamoto et al., 1998). After in situ hybridization, embryos were washed in benzyl benzoate/benzyl alcohol (2:1), mounted in Canada balsam/methyl salicylate (40:1), and photographed using a Zeiss Axiocam mounted on a Zeiss Axioplan microscope. Genomic DNA for genotyping was extracted by using the Qiagen DNeasy tissue kit. Primers and restriction enzymes used to genotype *dino*^{tt250} and *ntl*^{b160} were:

F dino 5′-ATTGTCTCAATCAGGTTGCTCC-3′,
R dino 5′-CGGGTTGGTTTTATTTGTAA-3′ (*Msp*1 restriction site polymorphism)
F:ntl 5′-GAAGTGACCACAAGGAAGTCC-3′
R:ntl 5′-ACGAACCCGAGGAGTGAACAG-3′ (*Alu*1 restriction site polymorphism)

Analysis of gene expression by real-time PCR

Total RNA was prepared from pools of 10 embryos using TRIzol reagent (Invitrogen). For each experiment, three pools of experimental samples and two pools of control samples were run. cDNA was synthesized from 0.5 μg RNA using the SuperScript First Strand Synthesis Kit (Invitrogen) in a volume of 10 μl. After the RT reaction, the volume of each cDNA sample was brought up to 100 μl in dH_2O. Real-time PCR was carried out using an ABI Prism 7700 sequence detection system (Applied Biosystems). The real-time PCR reactions were set up using 5 μl cDNA, 7.5 μl 2× SYBR Green I mastermix (Eurogenetec, Philadelphia, PA) and forward and reverse primers (100 nM final concentration each) in a final volume of 15 μl. All reactions were run with a melting temperature of 55°C. The sequences for the primer pairs are (forward primer/reverse primer): *β-actin*, GATTCGCTGGAGATGATG/GTCTTTCTGTCCCATACCAA; *bmp2*, TGGTGCAGGACTCTCACAC/TGGAGCACCTCTACAAGGAG; *bmp4*, CAAACACCACACCAAAAGTG/TCTGCGGTGGATATGAGTTC; *bmp7*, TGCAGCTCTTAGTGGAGACC/AAACGGCTGCTTATTCTGAG; *otx2*, CCACTTTCTACCTCCTCCTC/TAGGAAGTGGAACCAGCATA; *hoxb1b*, TT-AAACAAGCGCCAACCTTT/GTGGTGAAATTGGTGCGTAT; *gata2*, GCTGAATGTGTGAACTGTGGA/TGGCTTGATAAGGGGTCTGT; *gata3*, CCTGCGGACTTTACCACAAG/ACAGTTTGCGCATGAGGTC; *vox*, CTCATCTCCAAGCTTTTCAG/GAATTTGGTTCTGATTCTGC; and *eve1*, GGGTAGTCTCTCTGGGTTTT/GAATAGAGAGCTGGTTGTGG. Each sample was run in duplicate along with a negative (water) control. In order to compare expression levels between control and experimentally treated embryos, a dilution series of noninjected cDNA control were included for each primer set in each run. The dilution series was serially diluted in the following conditions: 1:0 cDNA:H_2O; 1:10 cDNA:H_2O; and 1:100 cDNA:H_2O. This dilution series allowed a standard curve to be made for each primer set in each reaction.

For the analysis of real-time PCR reactions, the duplicate Ct values for each sample were averaged together and the relative amount of RNA was determined by interpolating the sample Ct value to the standard curve. The relative amount of RNA was normalized to the relative amounts of the endogenous control (β-actin). The fold change was determined by comparing the normalized value of the experimental samples to the normalized value of the control samples. Data shown are the average of two independent experiments.

Results

Neural tissue develops in the absence of Chordin and mesoderm

Zebrafish maternal-zygotic *one-eyed pinhead* (MZ*oep*) mutants are defective in Nodal signaling and lack all trunk mesendoderm including the organizer, yet generate a broad, well-patterned neural plate (Gritsman et al., 1999; Sirotkin et al., 2000). While most organizer markers are not expressed in Nodal signaling mutants, expression of *chordin*, a potent neural inducer, is initiated in these embryos (Gritsman et al., 1999; Sirotkin et al., 2000). In

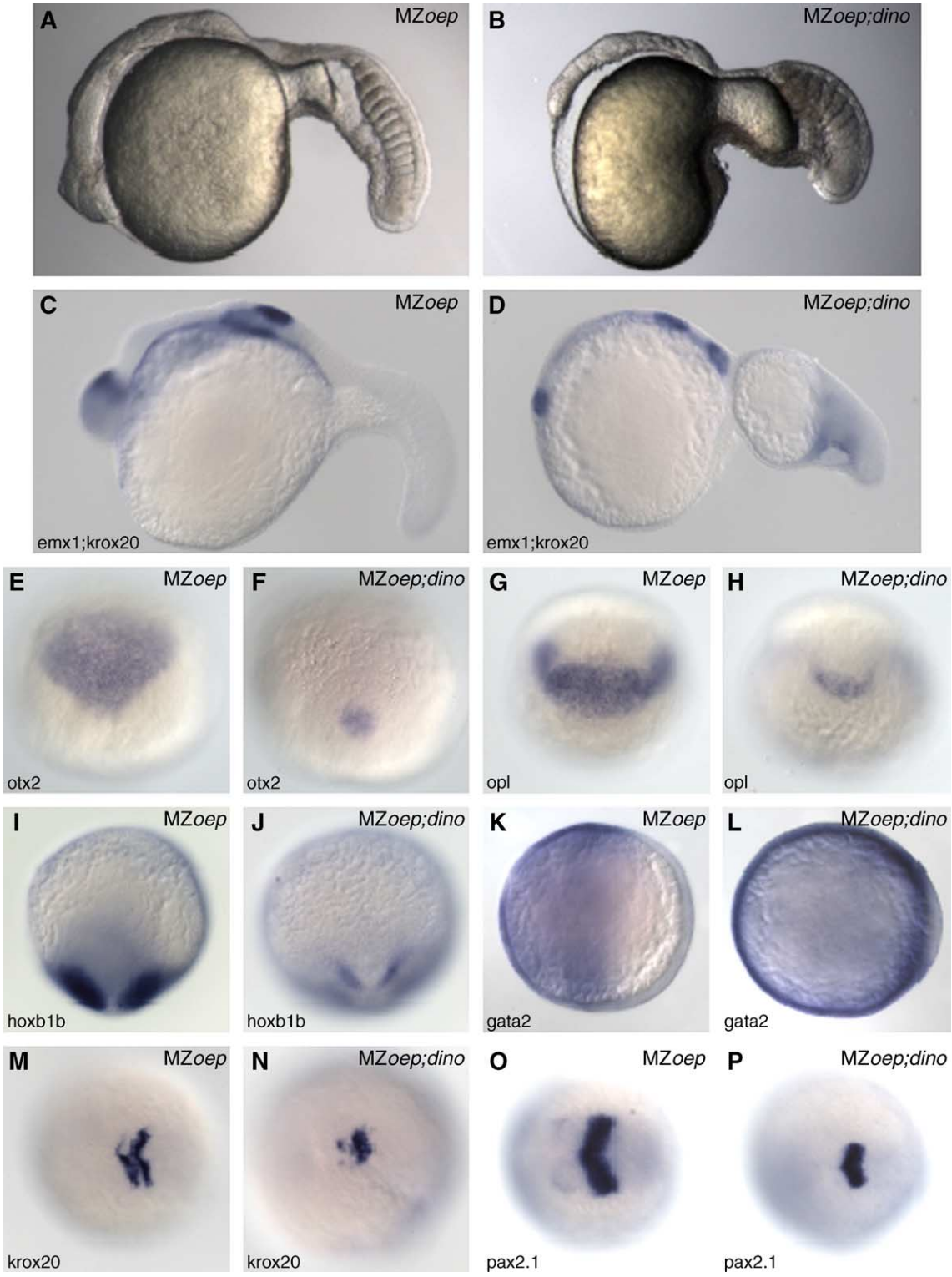


Fig. 1. Neural tissue is maintained in MZ*oep;dino* double mutants. Lateral views of living MZ*oep* and MZ*oep;dino* double mutants (A and B) at 22 h. The double mutant has reduced neural tissue and large tail somites compared to MZ*oep* single mutants. Analysis of the expression patterns of neural and presumptive epidermal markers in MZ*oep* and MZ*oep;dino* double mutants by whole mount RNA in situ hybridization. The presence of neural tissue at 22 h is confirmed by the expression of *emx1* and *krox20* (C and D). The expression domains of the anterior neural markers *otx2* and *opl* and the posterior neural marker *hoxb1b* are reduced in MZ*oep;dino* double mutants (F, H, and J) compared to MZ*oep* mutants (E, G, and I). The *gata2* expression domain is expanded dorsally in MZ*oep;dino* double mutants (K and L). During early somitogenesis, the neural plate is narrow in the double mutant as revealed by *krox20* and *pax2.1* expression (M–P). Panels C and D are lateral views; E–H are dorsal views at 70% epiboly (mid-gastrula); I and J are dorsal views at late gastrula (90% epiboly); K and L are animal pole views at 70% epiboly (mid-gastrula); and M–P are dorsal views at the three-somite stage. Genotypes of all embryos were determined following photography by PCR-based analysis.

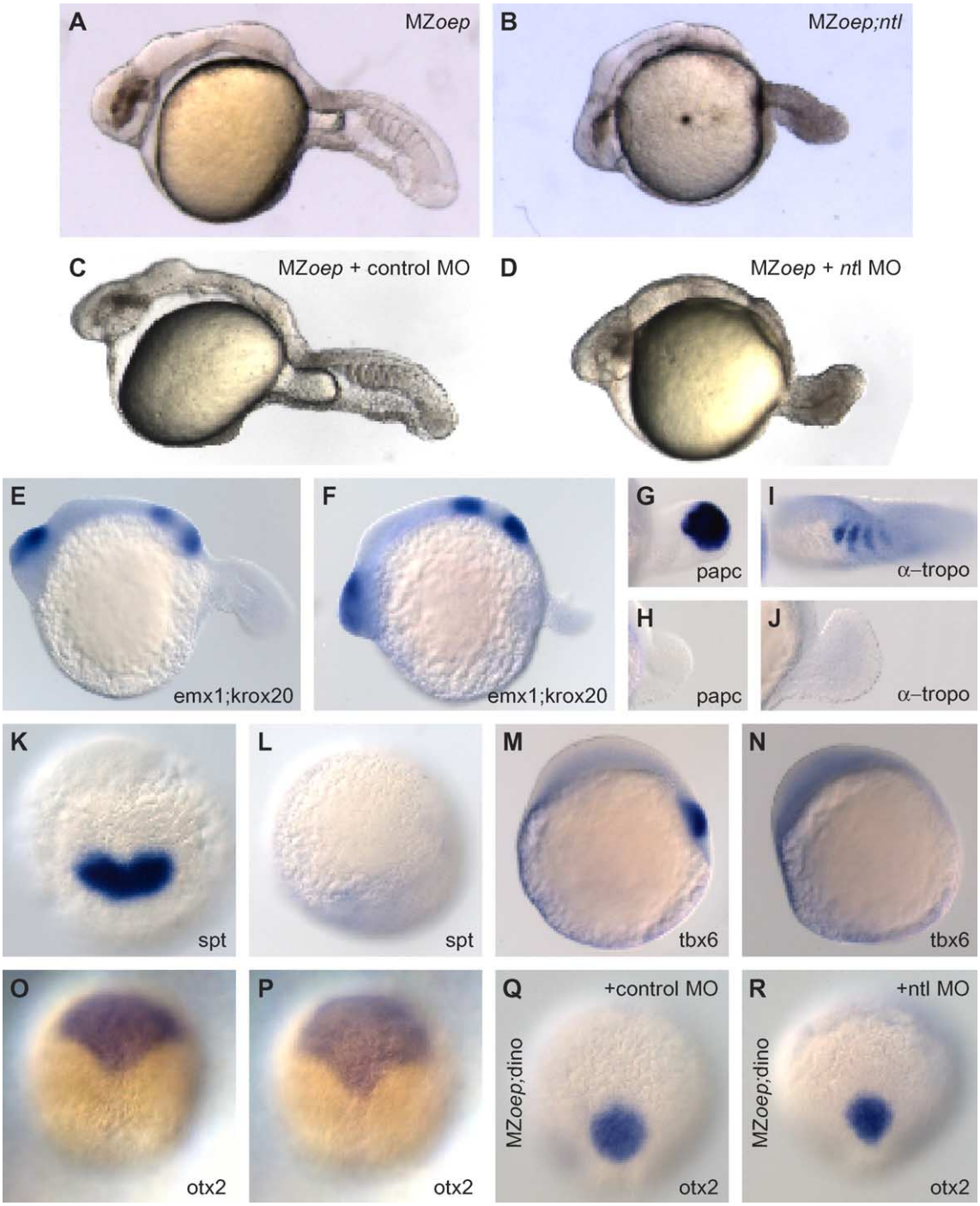


Fig. 2. The mesoderm is not required for neural induction. Lateral views of live MZ*oep* and MZ*oep;ntl* double mutant embryos (A and B). The double mutant lacks tail somites but forms anterior neural tissue. The double mutant phenotype can be phenocopied by microinjection of *ntl* morpholinos into MZ*oep* mutants (C and D). Analysis of the expression patterns of neural and mesodermal markers in MZ*oep* and MZ*oep;ntl* double mutants by whole mount RNA in situ hybridization. At 24 h, expression of *emx1* and *krox20* is indistinguishable in MZ*oep* (E) and MZ*oep;ntl* double mutant embryos (F). While MZ*oep* mutants express markers of tail mesoderm including *papc*, *α-tropomyosin*, *spt*, and *tbx6* (G, I, K, and M), these markers are all absent in MZ*oep;ntl* double mutant embryos (H, J, L, and N). Early expression of *otx2* during gastrulation is unaffected in MZ*oep;ntl* double mutant embryos (P) compared to MZ*oep* single mutants (O). The small *otx2* domain in MZ*oep;dino* embryos is not altered by treatment with *ntl* morpholinos (Q and R). A–F, 24-h embryos; G and H, lateral views of tails of 23 h, or, I and J, 24-h embryos; K and L, dorsal views of two-somite embryos, anterior is toward the top; M and N, lateral views of two-somite embryos; and O–R, dorsal views of mid-gastrula embryos (70% epiboly). Genotypes of all embryos were determined following photography by PCR-based analysis.

Xenopus, it has been suggested that expression of *chordin* at blastula stages within the dorsal animal cap is required for mesoderm-independent specification of anterior neural fates (Kuroda et al., 2004). Since the zebrafish *chordin* locus is disrupted in *dino* mutants (Schulte-Merker et al., 1997), we were able to determine if neural specification in the zebrafish Nodal signaling mutants is mediated by *chordin* by generating MZ*oep;dino* double mutants (Fig. 1).

MZ*oep;dino* double mutants have reduced neural tissue compared to MZ*oep* single mutants at 24 h (Figs. 1A–D). The double mutants also have fewer, broader tail somites than MZ*oep* single mutants. A similar posterior expansion is also observed in *dino* single mutants (Hammerschmidt et al., 1996; Schulte-Merker et al., 1997) and is a predicted result of excess BMP signaling. The expression domains of anterior neural markers (*otx2* and *opl*) and a posterior neural marker (*hoxb1b*) are dramatically reduced during gastrulation in the double mutant (Figs. 1E–J). At the same stages, *gata2* expression, which marks presumptive epidermis, is expanded in MZ*oep;dino* double mutants (Figs. 1K and L). During early somitogenesis, pax2.1 (midbrain) and krox20 (r3 and r5) are both correctly expressed in the double mutant, albeit in narrow domains (Figs. 1M–P). These results demonstrate that neural tissue is induced in MZ*oep;dino* double mutants and that the neural tissue undergoes correct anterior–posterior patterning. Because a small amount of neural tissue is present in MZ*oep;dino* double mutants, we can conclude that Chordin is not the sole signal responsible for specification of neural tissue in MZ*oep* mutants.

What signals in MZ*oep;dino* might account for the remaining neural tissue? Mesoderm is often considered a source of factors that neuralize the ectoderm. Because of the abnormal gastrulation movements in Nodal mutants, the location where prospective anterior neural tissue is specified is vegitally displaced by 90° compared to wild-type embryos (Gritsman et al., 1999). It is possible that in these mutants the dorsal ectoderm is exposed to signals from ventral (tail) mesoderm that may promote specification of the anterior neural domain. No-tail (ntl) is the zebrafish homologue of Brachyury and is required for specification of tail mesoderm (Halpern et al., 1993; Schulte-Merker et al., 1994). To determine whether the remaining tail mesoderm is the source of neuralizing signals in the absence of dorsal/trunk mesoderm, we generated MZ*oep;ntl* double mutants (Fig. 2).

From the earliest stages, MZ*oep* mutants show deficits in markers of trunk mesoderm but the posterior mesoderm is specified and tail somites form (Gritsman et al., 1999). In MZ*oep;ntl* double mutant embryos, no tail mesoderm is generated as evidenced by the absence of posterior mesodermal markers during early somitogenesis including *spadetail*, and *tbx6* and later tail markers including *papc* and *α-tropomyosin* (Figs. 2G–N). We therefore conclude that these embryos lack all mesoderm.

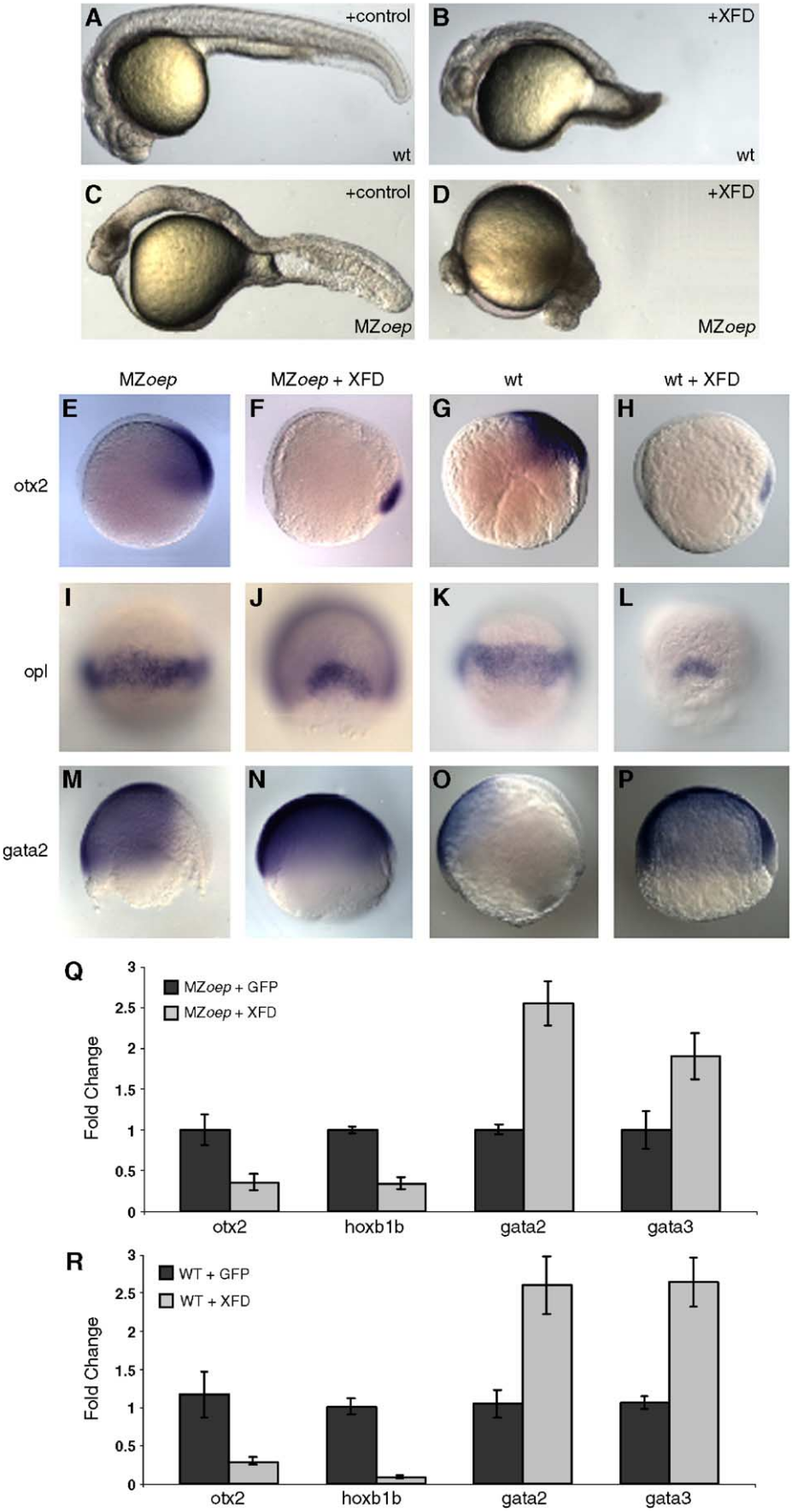
During gastrulation, expression of *otx2* in the anterior neural plate in MZ*oep;ntl* double mutants embryos is comparable to MZ*oep* single mutants (Figs. 2O and P). Likewise, at 24 h, the *emx* and *krox20* expression domains are similar to the domains in MZ*oep* embryos (Figs. 2E and F). Neural induction and patterning appear to be unaffected by the elimination of the remaining tail mesoderm in Nodal mutant embryos.

To determine whether Chordin masks a weak neural-inducing activity of factors originating in the tail mesoderm, we eliminated *ntl* function in MZ*oep;dino* double mutants using antisense morpholinos directed against the ntl translation start site (Nasevicius and Ekker, 2000). Microinjection of the morpholinos into MZ*oep* embryos phenocopies MZ*oep;ntl* double mutants (Figs. 2C and D). Treatment of MZ*oep;dino* double mutant embryos with *ntl* morpholinos does not alter expression of *otx2* during gastrulation (Figs. 2Q and R). Based on these results, we conclude that the tail mesoderm does not play a role in specification of the neural plate in MZ*oep* mutants.

FGF signaling is required for neural induction in zebrafish

While neural tissue is formed in the absence of mesoderm, signals that induce neural tissue may originate from marginal cells that are precursors to the mesoderm and endoderm in wild-type embryos. In Nodal mutants, these cells ultimately form neural tissue (Feldman et al., 2000). However, prior to the onset of gastrulation, these cells express markers characteristic of mesendodermal precursors including *wnt11*, *tbx6*, and *spt* (Gritsman et al., 1999; Mathieu et al., 2004). FGF signaling is also active in mesendodermal precursors and has been proposed to act as a neural inducer in some species (see Introduction). We reasoned that the activity of FGF in neural induction might

Fig. 3. XFD blocks neural induction in MZ*oep* mutants and wild-type embryos. Analysis of the expression patterns of ectodermal markers in control and XFD-injected MZ*oep* mutant embryos. Lateral views of 24-h live control-injected wild type (A) and MZ*oep* mutant (C) embryos and XFD-injected wild type (B) and MZ*oep* (D) embryos. XFD overexpression results in posterior defects in wild-type embryos but anterior neural deficits are apparent in XFD-injected MZ*oep* mutant embryos. Whole mount RNA in situ hybridization anterior neural and ectodermal markers in MZ*oep* and wild-type control (E, G, I, K, M, and O) and XFD (F, H, J, L, N, and P)-injected MZ*oep* mutant embryos and wild-type embryos at mid-gastrula (70% epiboly). The expression domains of the anterior neural markers *otx2* (E, F, G, and H) and *opl* (I, J, K, and L) are shifted toward the margin and dramatically reduced in XFD-injected embryos. The expression domains of *gata2* (M, N, O, and P) in the ventral ectoderm in XFD-treated embryos are expanded compared to control-injected embryos. For all microinjection experiments, 125 pg of XFD mRNA was injected. The consequence of XFD treatment on transcript levels of markers of neural tissue and presumptive epidermis in MZ*oep* mutants and wild-type embryos was monitored by real-time PCR (Q and R). The levels of neural markers *otx2* and *hoxb1b* are decreased in embryos microinjected with 125 pg XFD mRNA, while the levels of presumptive epidermal markers *gata2* and *gata3* are increased. The fold change (*y*-axis) of these markers is set relative to control (GFP)-injected embryos. Embryos were collected at mid-gastrulation (70% epiboly).


A
+control
wt
B
+XFD
wt
C
+control
MZoep
D
+XFD
MZoep
MZoep
MZoep + XFD
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wt + XFD
otx2
E
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G
H
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J
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gata2
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MZoep + GFP
MZoep + XFD
Fold Change
3
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WT + GFP
WT + XFD
Fold Change
otx2
hoxb1b
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be more apparent in the absence of dorsal mesoderm. Therefore, we sought to determine whether FGF signaling is required to generate the neural tissue that is observed in MZ*oep* mutants.

We first blocked FGF signaling by overexpressing the dominant-negative FGF receptor XFD (Amaya et al., 1991) in MZ*oep* mutant embryos. XFD has been used to block FGF signaling in both *Xenopus* and zebrafish embryos (Amaya et al., 1991; Griffin et al., 1995; Kroll and Amaya, 1996). The primary deficits reported in these experiments were the elimination of trunk and tail mesoderm. Deficits were not observed in the formation of anterior neural tissue. However, microinjection of XFD into MZ*oep* mutants severely reduced the amount of neural tissue present at 24 h compared to control-injected MZ*oep* mutants embryos (Figs. 3C and D). Microinjection of XFD into wild-type embryos resulted in elimination of trunk and tail mesoderm. However, as in previous experiments, anterior neural tissue appeared intact at 24 h (Figs. 3A and B). These experiments demonstrate that XFD treatment has markedly different effects on wild type and MZ*oep* mutant embryos.

To understand the different consequences of blocking FGF signaling in Nodal mutants and wild-type embryos, we sought to determine if XFD inhibits neural induction in MZ*oep* mutants. If FGF signaling is required for neural induction, impeding FGF signaling will inhibit expression of neural markers during gastrulation and enhance expression of markers of presumptive epidermis. At mid-gastrula stages, the expression of the anterior neural markers *otx2* (Figs. 3E and F), *opl* (Figs. 3I and J), and *cyp26* (data not shown) are all reduced in MZ*oep* mutants after XFD treatment when assayed by RNA in situ hybridization. During gastrulation, the *gata2* (Figs. 2M and N) and *gata3* (data not shown) expression domains in the presumptive epidermis are greatly expanded by XFD treatment.

The expression of neural and nonneural ectoderm markers was also examined in mid-gastrula stage MZ*oep* embryos using real-time PCR. Consistent with the RNA in situ hybridization results, XFD-treated MZ*oep* embryos show dramatic decreases in expression levels of the neural markers compared to controls. In control-injected embryos, the expression levels of *otx2* and *hoxb1b* were >4 times higher than embryos injected with XFD (Fig. 3Q). Conversely, XFD-treated embryos expressed *gata2* and *gata3* at levels ~2.5 times greater than control-injected embryos (Fig. 3Q). These results show that blocking FGF signaling with XFD in MZ*oep* mutants promotes expression of markers of presumptive epidermis at the expense of expression of neural markers. Therefore, we conclude that

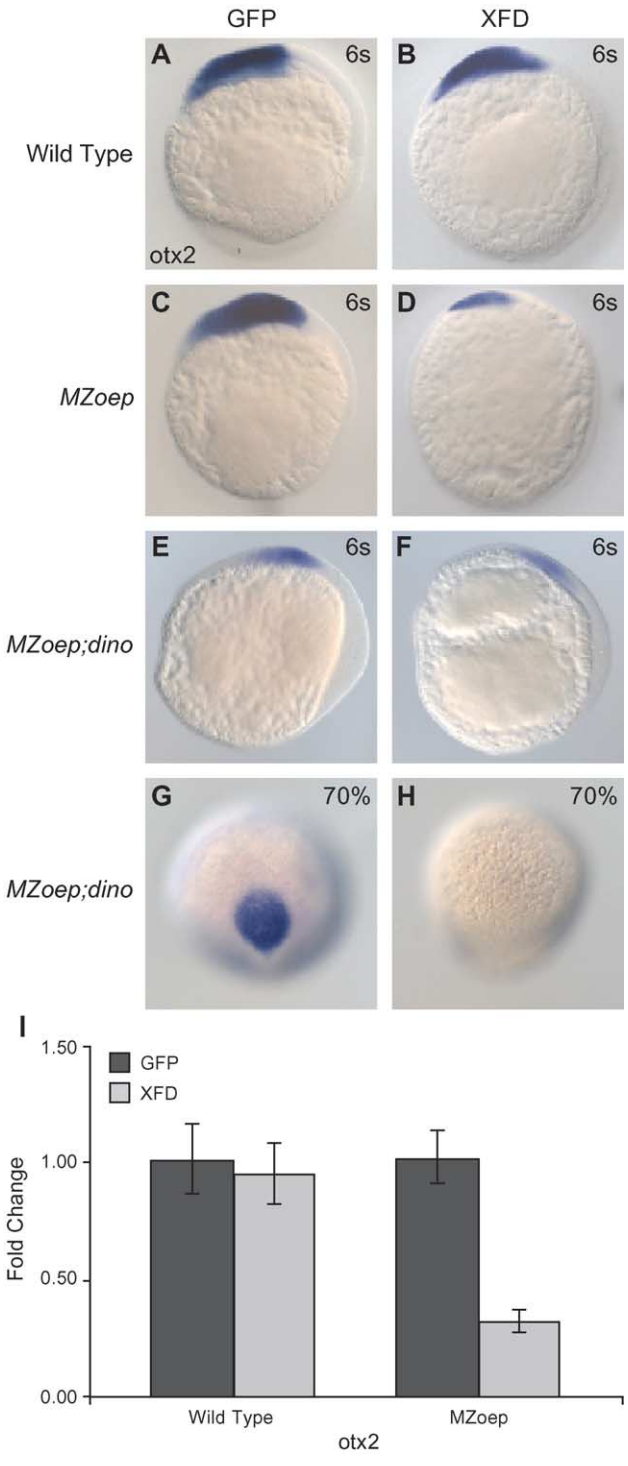


Fig. 4. Recovery of anterior neural tissue following FGF inhibition depends on Nodal signaling. (A–H) otx2 in situ hybridization of wild type, MZ*oep*, and MZ*oep; dino* embryos microinjected with 125 pg XFD or GFP mRNA. Expression of *otx2* is comparable at the six-somite stage in wild-type embryo microinjected with GFP or XFD mRNA (A and B), while XFD-injected MZ*oep* embryos have a reduced *otx2* domain (C and D). The *otx2* domain is comparable in XFD- and GFP-injected MZ*oep*;*dino* mutant embryos at the six-somite stage (E and F), while *otx2* expression is eliminated in XFD-injected MZ*oep; dino* embryos at 70% epiboly (mid-gastrula) (G and H). Stages of embryos are indicated. Panels A–F are lateral views, G and H are dorsal views. (I) Real-time PCR analysis of *otx2* transcript levels in wild type and MZoep XFD-injected embryos at the six-somite stage. The fold change (*y*-axis) of these markers is set relative to control (GFP)-injected embryos. XFD-injected MZoep embryos show a decrease in *otx2* expression while XFD-injected wild-type embryos show no change in *otx2* expression compared to controls.

FGF signaling mediates specification of the neural domain in MZ*oep* mutants.

Since impeding FGF signaling severely reduces anterior neural tissue in MZ*oep* mutants during gastrulation, we wanted to determine why XFD-treated wild-type embryos generate overtly wild-type anterior neural structures by 24 h. One possibility is that the deficits produced by inhibition of FGF signaling in MZ*oep* mutants are not apparent in wild-type embryos due to redundant activity of the organizer (dorsal mesoderm). To test this hypothesis, we assayed ectodermal markers in wild-type gastrula stage embryos following inhibition of FGF signaling. These treatments produced clear deficits in neural specification.

Expression of anterior neural markers including *otx2* (Figs. 3G and H), *opl* (K and L), and *cyp26* (data not shown) during gastrulation was assayed using RNA in situ hybridization. The expression domains of these markers were greatly reduced and shifted toward the margin. At the same stages, the expression domains of *gata2* (Figs. 3O and P) and *gata3* (data not shown) in the presumptive epidermis were expanded. We also measured the effects of inhibiting FGF signaling with XFD on neural induction in wild-type embryos at mid-gastrula stages using real-time PCR (Fig. 3R). Control-injected embryos expressed the neural markers *otx2* and *hoxb1b* at levels nearly four times higher than XFD-injected embryos. Conversely, XFD-treated embryos expressed the markers of presumptive epidermis *gata2* and *gata3* at about twice the levels of control-injected embryos.

In addition to using XFD to block FGF signaling, we also blocked signaling with Δ-FGFR-4 (Hongo et al., 1999). XFD is a dominant-negative FGF type 1 receptor and Δ-FGFR-4 is a dominant-negative type 4 receptor. Since FGF receptors form heterodimers, each of these reagents may block signaling through multiple receptors. Like XFD-treated embryos, Δ-FGFR-4-treated embryos show a decrease in neural markers and an expansion of markers of presumptive epidermis at mid-gastrula stages (data not shown). Coinjection of XFD and Δ-FGFR-4 produces effects on the neural plate similar to either construct alone (data not shown). This suggests that both constructs block similar signaling events required for generation of the neural plate.

These results show that inhibiting FGF signaling in wild-type embryos inhibits the expression of neural markers and enhances expression of markers of the presumptive epidermis during gastrulation. Since expression levels of these markers is indicative of cell fate choice within the ectoderm, we conclude that in zebrafish FGF signaling mediates ectodermal cell fate decisions and is required for neural induction.

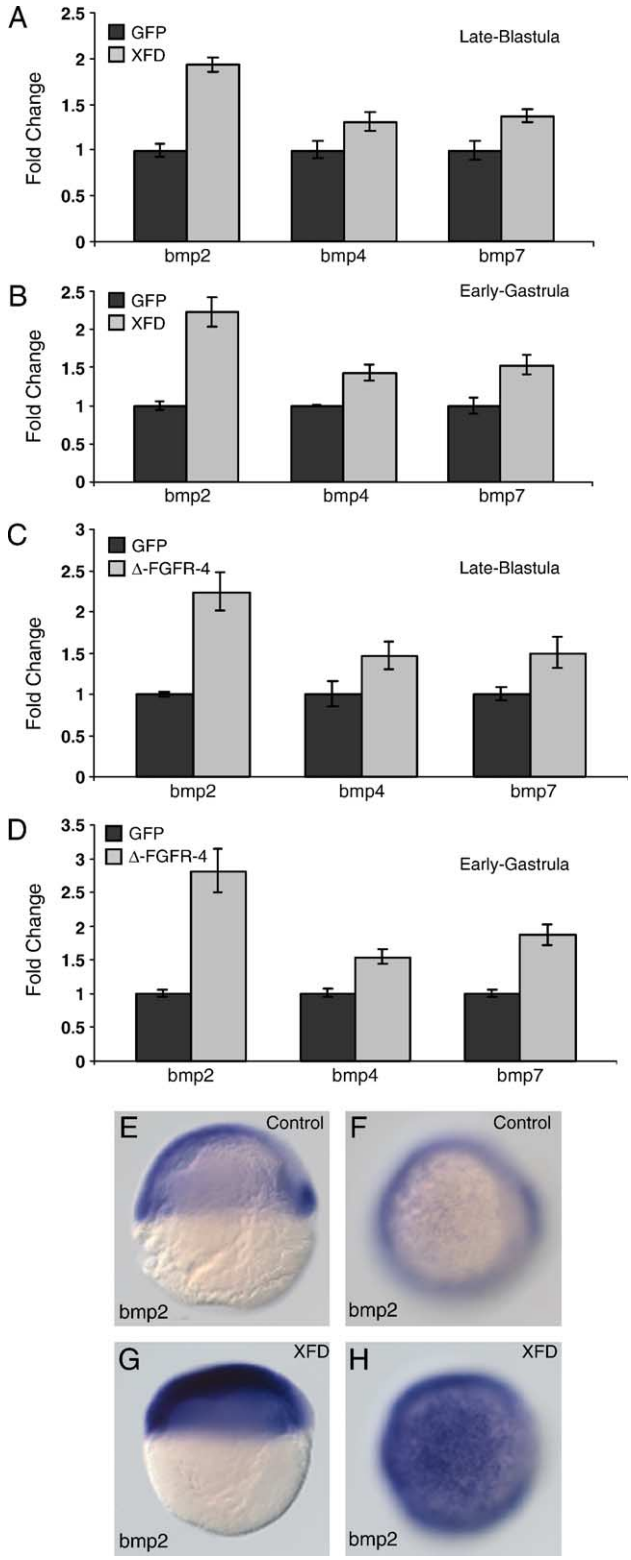


Fig. 5. FGF signaling represses BMP transcription. Real-time PCR analysis of XFD (A and B) and Δ-FGFR-4 (C and D)-microinjected wild-type embryos during late blastula and early gastrula stages. The fold change (*y*-axis) of the markers *bmp2*, *bmp4*, and *bmp7* is set relative to control (GFP)-injected embryos. XFD- and Δ-FGFR-4-injected embryos show an increase BMP transcript levels during late blastula stages. (E–H) RNA in situ hybridization of *bmp2* expression in control (E and F)- and XFD-injected wild-type embryos (G and H). In XFD-injected embryos, the *bmp2* expression domain extends into the dorsal ectoderm. Panels E and G are lateral views of shield stage embryos and F and H are animal pole views of the same embryos.

Recovery of anterior neural tissue following inhibition of FGF signaling requires Nodal signaling

Since both wild type and MZ*oep* mutants show anterior neural deficits during gastrulation following inhibition of FGF signaling, but only MZ*oep* mutants show anterior neural defects at 24 h (Figs. 3A–D), wild-type embryos must be able to generate neural tissue after mid-gastrula stages following inhibition of FGF signaling, but MZ*oep* embryos cannot. To determine the time at which the neural tissue recovers in wild-type embryos, we performed RNA in situ hybridization using an *otx2* antisense probe at various time points during late gastrula stages and early somitogenesis. We found that by the six-somite stage, expression of *otx2* is comparable in control and XFD-injected wild-type embryos (Figs. 4A and B). In contrast, MZ*oep* embryos injected with XFD have diminished expression of *otx2* at the six-somite stage (Figs. 4C and D). *otx2* expression was also measured by real-time PCR in both wild-type and MZ*oep* XFD-injected embryos at the six-somite stage (Fig. 4I). In agreement with the in situ hybridization data, *otx2* expression levels, wild-type embryos recover to control levels following XFD treatment, while MZ*oep* embryos fail to recover. These results suggest that the recovery of neural tissue in wild-type embryos with compromised FGF signaling depends on Nodal signaling.

Synergy between FGF signaling and Chordin in neural specification

To determine whether there is redundancy in the activities of FGF signaling, Chordin, and additional mesodermal factors in neural induction, we blocked FGF signaling using XFD in MZ*oep;dino* double mutants. At mid-gastrulation, *otx2* is not expressed in XFD-treated MZ*oep;dino* double mutant embryos (Figs. 4G and H). However, in these embryos, *otx2* is expressed at low levels by the six-somite stage (Figs. 4E and F). From these results, we conclude that the expression of neural markers at mid-gastrula stages requires FGF signaling, Chordin, and downstream targets of Nodal signaling (mesoderm). Subsequent expression of neural markers may depend on additional late acting factors or may be the result of an inability of microinjected XFD RNA to completely repress FGF signaling at later stages.

FGF signaling represses transcription of BMPs

Since FGF activity has been shown to repress BMP transcript levels in the chick, we tested the hypothesis that the effects of blocking FGF signaling on expression of neural markers could be traced to a direct impact on BMP transcript levels. Three zebrafish BMP family members are expressed during the late blastula and early gastrula periods, *bmp2*, *bmp4*, and *bmp7* (Kishimoto et al., 1997; Schmid et al., 2000). All of these molecules have been shown to regulate epidermal cell fate choices within the ectoderm. Overwhelming evidence suggests that inhibition of BMP signaling promotes expression of neural makers and represses epidermal fates (Munoz-Sanjuan and Brivanlou, 2002; Stern, 2002).

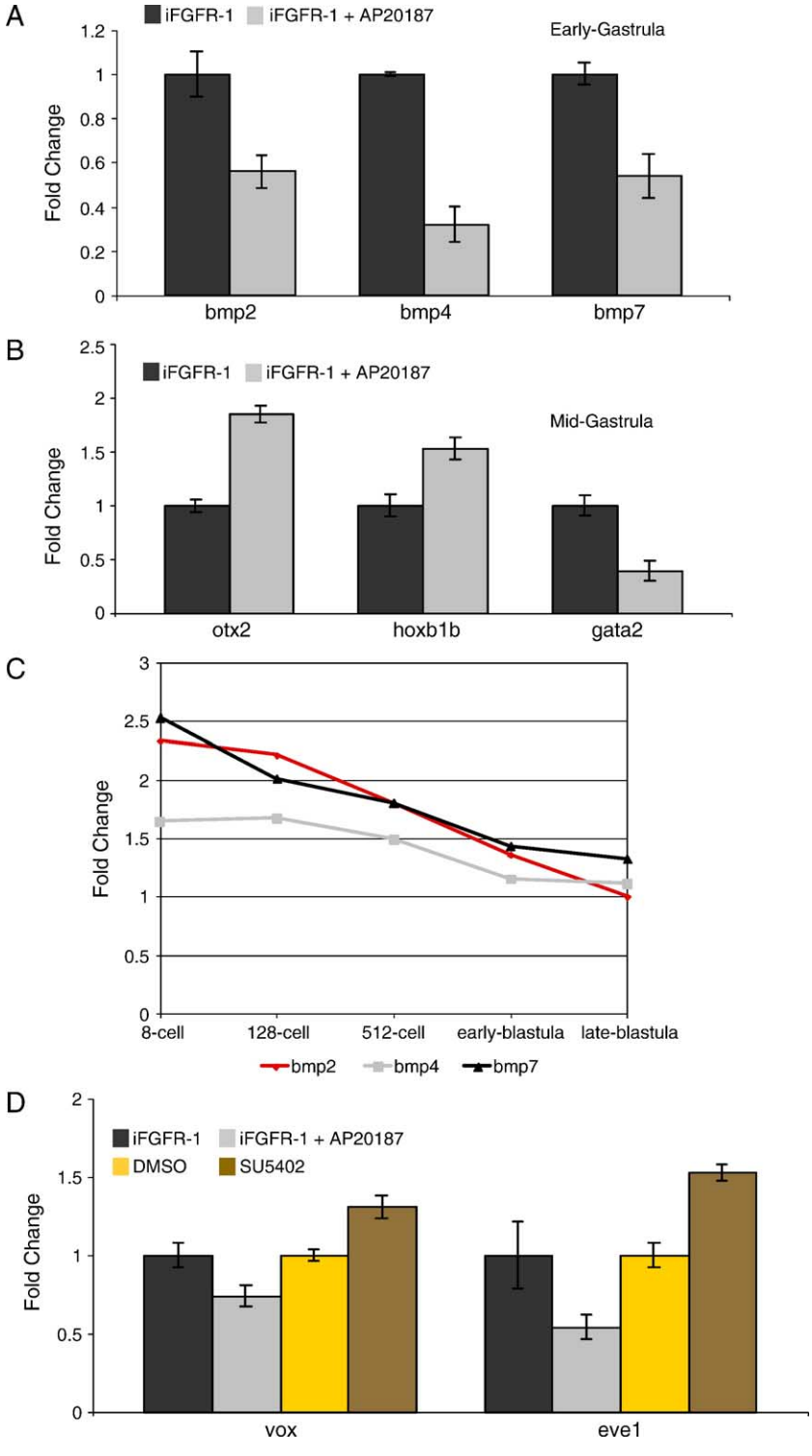
To determine the role of FGF signaling in regulating BMP transcription, we analyzed the effects of inhibiting FGF signaling on the expression of BMP ligands. Microinjection of XFD or Δ-FGFR-4 at the one- to four-cell stage resulted in increased levels of *bmp2*, *bmp4*, and *bmp7* transcripts during late blastula and early gastrulation stages as assayed by real-time PCR (Fig. 5). During the late blastula period, XFD and Δ-FGFR-4 injected embryos show almost twice the levels of *bmp2* transcripts, and modest increases in *bmp4* and *bmp7* transcript levels, as compared to control-injected embryos (Figs. 5A and C). By early gastrulation, the effects of both treatments on *bmp4* and *bmp7* transcription are more evident, although not as substantial as the effect on *bmp2* transcript levels (Figs. 5B and D).

To determine whether the increases in *bmp* transcript levels correlate with an expansion of *bmp* transcripts into the dorsal BMP-free zone that gives rise to the neural plate, we examined *bmp* expression after XFD microinjection by whole mount RNA in situ hybridization (Figs. 5E–H). In XFD-treated embryos, *bmp2* (Figs. 5E–H) and *bmp4* (data not shown) transcripts extend further dorsal than in control-injected embryos. Taken together, both the RNA in situ hybridization and real-time PCR data show that inhibition of FGF signaling enhances BMP transcript levels.

Fig. 6. FGF signaling is required during the late blastula period to regulate BMP transcription. Embryos were injected with iFGFR-1 and activated with AP20187 at the 512-cell stage. Embryos were collected at early and mid-gastrula stages for real-time PCR. Embryos at early gastrulation (shield stage) (A) were tested for their fold change (*y*-axis) of *bmp2*, *bmp4*, and *bmp7* (A), and for their expression of *otx2*, *hoxb1b*, and *gata2* (B) at mid-gastrulation (70% epiboly). Activating FGF signaling at the 512 cell stage results in an early decrease in *bmp2*, *bmp4*, and *bmp7* expression at shield stage. At mid-gastrulation, these embryos have increases in *otx2* and *hoxb1b* transcript levels and a decrease in *gata2* levels. (C) FGF signaling was inhibited with the pharmacological drug SU5402 at 8-cell, 128-cell, 512-cell, early blastula, and late blastula, and collected at shield stage (early gastrula) for real-time PCR analysis. Graphed is the fold change (*y*-axis) for *bmp2*, *bmp4*, and *bmp7* levels relative to control-treated embryos. These results show that the effectiveness of the SU5402 treatment in blocking repression of BMP transcript levels diminishes between the 512-cell and early blastula (sphere stages). (D) FGF signaling was either inhibited or activated with SU5402 or AP20187 at the 512-cell stage. Embryos were collected at the shield stage (early gastrula) for their fold change (*y*-axis) of *vox* and *eve1*. Activating FGF signaling results in a 25% and 50% reduction in transcript levels for *vox* and *eve1*, while inhibiting FGF results in a 30% and 50% increase in *vox* and *eve1* transcript levels. These results show that inhibiting or activating FGF at the 512-cell stage results in loss of BMP target expression.

FGF signaling regulates blastula stage BMP transcript levels

Our results demonstrate that microinjection of mRNAs that block FGF signaling into one- to four-cell embryos results in late blastula and early gastrula stage increases in BMP transcript levels. To more precisely determine when FGF signaling is required, we used reagents that allowed activation or repression of FGF signaling at specific stages. To control activation of FGF signaling, we utilized an inducible FGF type I receptor (iFGFR-1) (Pownall et al., 2003; Welm et al., 2002). This construct contains two mutant FKBP12 dimerization domains fused to the carboxy terminus of the FGFR1 receptor. A small synthetic molecule AP20187 induces dimerization of the FKBP12 domains and FGF receptor activation. To block FGF signaling at specific



stages of development, we used a pharmacological inhibitor of FGF SU5402 (Mohammadi et al., 1996). Both methods allowed us to activate or inhibit FGF signaling at controlled time points throughout development.

To determine the effect of activation of FGF signaling on BMP transcript levels, we utilized iFGFR-1. FGF receptor activation decreases early gastrula stage expression of BMP ligands (Fig. 6). Embryos were injected with iFGFR-1 and treated with AP20187 or control media just prior to the mid-blastula transition (512-cell stage). These embryos were allowed to develop to early and mid-gastrula stages. At shield stage (early gastrula), embryos were collected and *bmp* transcript levels were monitored using real-time PCR. Activation of FGF signaling at 512-cell stage reduced the levels of all three *bmp* transcripts at shield stage to about 50% wild-type levels (Fig. 6A). By late gastrulation, this treatment resulted in increases in the transcript levels of the neural markers *otx2* and *hoxb1b* and a corresponding decrease in the transcript levels of the presumptive epidermal marker *gata2* (Fig. 6B). These results demonstrate that activation of FGF signaling during the late blastula and early gastrula stages reduces levels of *bmp* transcripts and enhances expression of early neural markers at the expense of presumptive epidermal markers.

To determine the stages when FGF signaling is required to repress BMP transcript levels, embryos were treated with the FGF inhibitor SU5402 at 8-cell, 128-cell, 512-cell, sphere, and 30% epiboly stages, and collected at shield stage (early gastrula). The relative *bmp* expression levels were monitored by real-time PCR. Like XFD and Δ-FGFR-4, early SU5402 FGF inhibition results in an increase in *bmp2*, *bmp4*, and *bmp7* expression at late blastula and early gastrula stages (Fig. 6C). Inhibiting FGF signaling prior to the mid-blastula transition (8, 128, or 512 cell stages) results in an increase in *bmp2*, *bmp4*, and *bmp7* transcript levels at shield stage (early gastrula). However, by sphere stage (late blastula), the ability of SU5402 to increase BMP transcript levels at shield stage decreases substantially (Fig. 6C). These results demonstrate that FGF signaling acts between the 512-cell stage and sphere stage to repress BMP transcript levels and suggest that the FGF ligands responsible for this activity are likely to be encoded by early zygotic genes.

To determine whether FGF also regulates early expression of BMP target genes, we monitored *vox* and *eve1* (Joly et al., 1993; Melby et al., 2000) expression levels after manipulating FGF signaling. FGF signaling was inhibited or activated using the SU5402 or iFGFR-1/AP20187 methods at the 512-cell stage. Samples were then collected at shield stage for real-time PCR for *vox* and *eve1*. Inhibiting FGF results in about a 30% increase in *vox* and a 50% increase in *eve1* expression (Fig. 6D). Conversely, activating FGF signaling results in 25% and 50% reduction in *vox* and *eve1*, respectively. These results suggest that prior to early gastrula stages FGF acts to diminish output of the BMP pathway.

Early FGF regulation of BMP transcription is independent of Chordin

Repression of BMP transcript levels might be caused by disruption of BMP autoregulation since BMP signaling induces transcription of BMP ligands in an autoregulatory loop (Hild et al., 1999; Jones et al., 1992). Alternatively, FGF signaling might act to repress the initial transcription of BMPs. Either mechanism could account for the effects of FGF on ectodermal cell fate choices.

One mechanism of action of FGF to regulate *bmp* expression could be via controlling the expression of the BMP antagonist Chordin. Chordin inhibits autoregulation of BMP expression by blocking BMP signaling. Activation of the FGF pathway with iFGFR-1 or FGF3 mRNA induces *chordin* expression (Fig. 7B and data not shown; and Koshida et al., 2002). More importantly, suppressing FGF signaling with XFD sharply reduces expression of *chordin* at the start of gastrulation (Figs. 7C and D) and throughout the gastrula period (data not shown). These results demonstrate that FGF signaling is essential for *chordin* expression.

It has been suggested that the activity of FGF3 in regulating neural marker expression in late gastrula embryos is abolished in *dino* mutants (Koshida et al., 2002). To determine whether FGF acts via Chordin to repress *bmp* transcript levels, we injected iFGFR-1 mRNA (Figs. 7E–H) or fgf3 mRNA (Figs. 7I–L) into embryos from *dino* heterozygote intercrosses. Embryos were subjected to RNA in situ hybridization with *bmp4* or *bmp2* probes. As in our earlier experiments, activation of FGF signaling with either reagent resulted in decreases in *bmp* transcript levels in wild-type embryos. Activation of FGF signaling in *dino* mutants also resulted in decreases in *bmp* transcript levels (Figs. 7G and H, and K and L). Furthermore, overexpression of *chordin* does not impact blastula stage expression of BMPs (Fig. 7M). These results suggest that while induction of *chordin* transcription is one means by which FGF can repress BMP signaling and BMP transcript levels by autoregulation, this mechanism does not account for the late blastula stage clearing of BMP transcripts from the dorsal ectoderm.

FGF does not require protein synthesis to inhibit BMP signaling during the late blastula period

To further address the means by which activation of FGF signaling represses *bmp* transcript levels, we investigated whether FGF-mediated repression requires protein synthesis. We activated FGF signaling just prior to the MBT using the iFGFR-1 construct and placed embryos in cycloheximide to block translation. Samples were placed in AP20187, cycloheximide, or both reagents, and collected embryos after 1 h. BMP transcript levels were then examined by real-time PCR (Fig. 8). Treatment of embryos with cycloheximide resulted in

greater than 2-fold increase in levels of each of the *bmp* transcripts compared to control embryos. But when FGF signaling was activated in the presence of cycloheximide, the expression levels of *bmp2*, *bmp4*, and *bmp7* returned to near baseline levels. Activation of FGF signaling with AP20187 in the absence of cycloheximide results in a

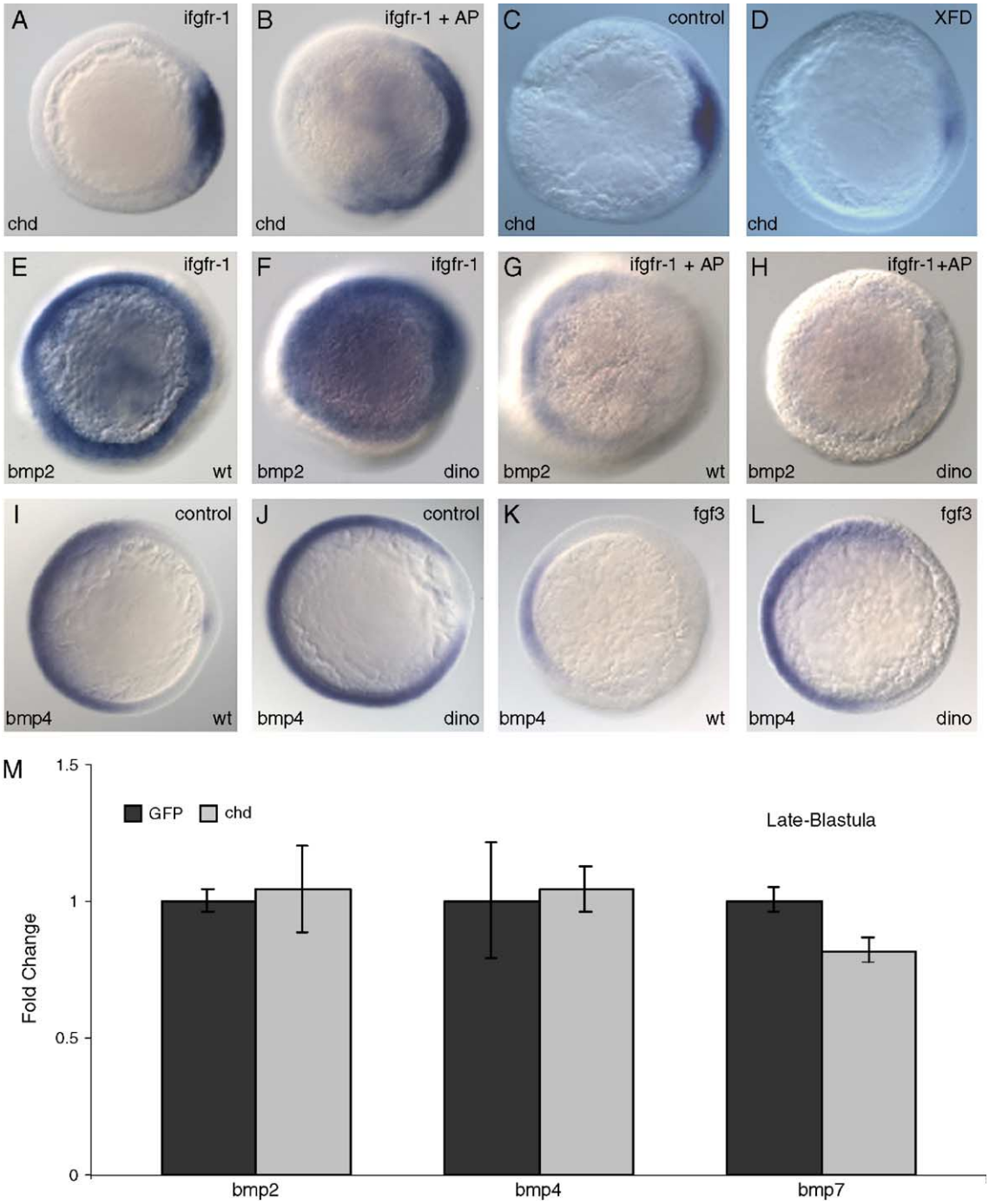


Fig. 7. FGF regulates *chordin* expression but does not require Chordin to repress BMP transcript levels. Addition of AP20187 to iFGFR-1-injected embryos induces *chordin* expression (B) throughout the embryo while in the absence of AP20187 iFGFR-1 embryos maintain wild-type levels of *chordin* (A) at shield stage (early gastrula). Microinjection of 125 pg XFD mRNA has the opposite effect and dramatically reduces *chordin* expression at shield stage (D) compared to control-injected embryos (C). Whole mount RNA in situ hybridization of embryos from a *dino* heterozygote intercross (E–L). *bmp2* expression at shield stage in iFGFR-1-injected embryos (E–H) exposed to AP20187 (G and H) or embryo water (E and F). The *dino* mutation does not suppress the activity of iFGFR-1 to repress bmp transcript levels. Genotypes of embryos are indicated. (I–L) *bmp4* expression at shield stage in control (I and J) or embryos injected with 50 pg *fgf3* mRNA-injected embryos (K and L). All views are animal pole. Genotypes of embryos were determined by PCR-based genotyping following photography. To determine if chordin has an effect on BMP transcript levels, real-time PCR was performed on embryos injected with 100 pg *chordin* and collected at 30% epiboly (M). Graphed is the fold change (y-axis) for *bmp2*, *bmp4* and *bmp7* levels relative to control injected embryos. These results show that *chordin* overexpression does not impact BMP transcript levels during the blastula period.

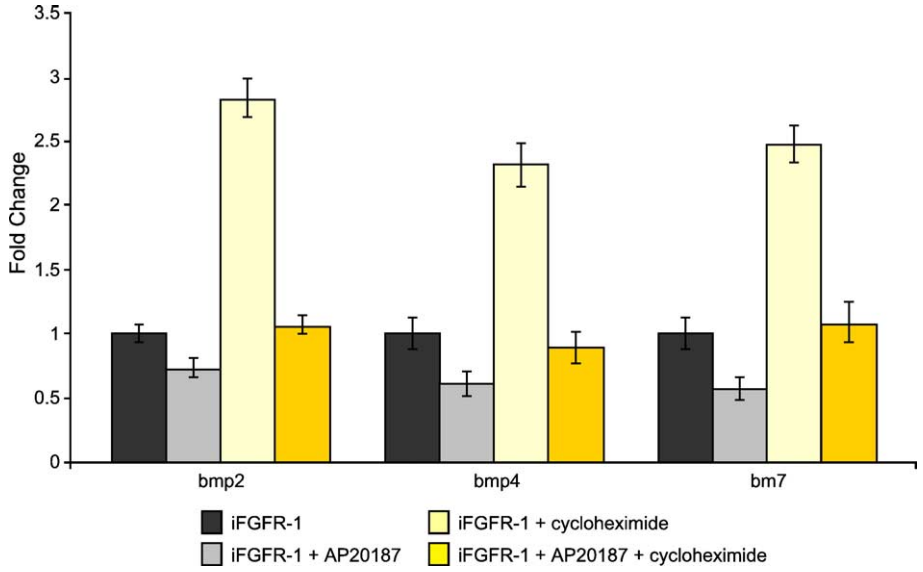


Fig. 8. FGF signaling does not require protein synthesis to inhibit BMP signaling. Embryos were injected with iFGFR-1 and treated with AP20187, cycloheximide, or both at the 512-cell stage. Embryos were collected after 1 h for real-time PCR analysis. The fold change (*y*-axis) of the BMP markers *bmp2*, *bmp4*, and *bmp7* is graphed relative to control (iFGFR-1). These results show that protein synthesis is not required for FGF signaling to inhibit BMP transcript levels.

30–50% decrease in *bmp2*, *bmp4*, and *bmp7* transcript levels. These results demonstrate that FGF signaling does not require protein synthesis to repress BMP transcript levels during the late blastula period.

Discussion

The early neural domain is established by the combined actions of Chordin and the Nodal and FGF signaling pathways

The vertebrate ectoderm is partitioned into neural and nonneural territories as a result of inhibition of BMP signaling. The mechanisms by which the BMP-free zone is established are complex, and investigators working on various model organisms have focused on different mechanisms. In this study, we investigated the interplay between Chordin, the Nodal pathway, which is required for induction of mesoderm, and the FGF pathway. Our results demonstrate that all three contribute to the establishment of the neural territory.

As a means of identifying endogenous neural inductive signals, we sought to determine the signals that generate neural tissue in Nodal signaling mutants. These embryos lack trunk mesendoderm and markers of dorsal mesoderm except *chordin*, yet have well-patterned neural tissue despite undergoing abnormal morphogenesis. Since Chordin is a potent neural inducer, we evaluated the role of Chordin in neural induction in the absence of dorsal mesoderm by generating MZ*oep;dino* double mutants. Neural markers are still expressed in these double mutants, although in restricted territories (Fig. 1). This result suggests that although Chordin plays a substantial role in specification of neural tissue in MZ*oep* mutants (and in wild-type embryos), additional factors must be involved.

Recent experiments in *Xenopus* have suggested that Chordin is required for neural induction in the absence of mesoderm (Kuroda et al., 2004). Kuroda et al. identified an early population of dorsal ectodermal cells that express *chordin* and *noggin* and give rise to parts of the CNS. MZ*oep;dino* double mutants also lack Noggin since its expression also depends on Nodal signaling (Sirotkin et al., 2000; and data not shown). These findings may reflect species-specific mechanisms or be due to differences in the combined effects of CerS and *chd* morpholinos compared to MZ*oep;dino* double mutants. However, it is plausible that the neural tissue we observe in the absence of mesoderm and Chordin is a result of autonomous signals from the ectoderm as observed in the Kuroda et al. study.

Since the mesoderm is thought to be a key source of neural inductive signals, we also assayed the ventral mesoderm (tail), which is maintained in the absence of Nodal signaling as a possible source of neural inducing signals. Unlike in wild-type embryos, cells that are fated to become tail mesoderm are positioned adjacent to the dorsal ectoderm in MZ*oep* mutants (Carmany-Rampey and Schier, 2001). MZ*oep* single mutants lack mesodermal markers during gastrulation but do ultimately form tail somites (Gritsman et al., 1999). No mesoderm is specified in MZ*oep;ntl* double mutants as demonstrated by the absence of mesodermal markers during somitogenesis (Fig. 2). Neural specification is unaffected in these mutants as evidenced by maintenance of *otx2* expression in a domain comparable to MZ*oep* single mutants (Figs. 2O and P). Since these embryos still express *chordin*, we sought to determine whether tail mesoderm and

Chordin have redundant roles in neural specification by eliminating the remaining ventral mesoderm in MZ*oep;dino* double mutants using *ntl* morpholinos. This treatment did not alter the *otx2* expression domain of MZ*oep;dino* double mutant embryos (Figs. 2Q and R). From these experiments we conclude that factors in addition to Chordin and molecules found in the mesoderm are required for the specification of anterior neural tissue.

However, despite the failure of mutant marginal cells to become differentiated mesoderm in our experiments, it is likely that these cells retain some signaling capabilities characteristic of the mesoderm. In fact, the expression of *krox20* demonstrates that the signals that posteriorize the neural plate to form the hindbrain are still present in these mutant embryos. These posteriorizing signals are thought to originate from somite precursors (Stern et al., 1991; Woo and Fraser, 1997). The maintenance of this signaling activity implies that mutant marginal cells retain some activities characteristic of mesendodermal precursors.

An FGF-mediated signal may account for such an activity in mesendodermal precursors. Studies in the chick have implicated an early role for FGF signaling in defining the neural territory (Wilson et al., 2000, 2001). However, experiments on the role of FGF in neural induction in amphibians and zebrafish have produced conflicting conclusions (Hongo et al., 1999; Kroll and Amaya, 1996; Pera et al., 2003; Ribisi et al., 2000). While recent experiments have suggested that FGFs act as neural inducers, 1- to 2-day frog or fish embryos that have been treated with XFD (Amaya et al., 1991; Griffin et al., 1995) or Δ-FGFR-4 (Hongo et al., 1999 and unpublished result) often still have eyes and well-patterned anterior neural tissue. Therefore, we sought to carefully examine the effects of the role of FGF in neural cell fate choice in zebrafish.

Our analysis of the effects of blocking FGF signaling in Nodal mutants and wild-type embryos clearly demonstrates that FGF signaling is required for proper neural and epidermal cell fate choice within the ectoderm. Inhibiting FGF signaling in both Nodal mutants and wild-type embryos led to a decrease in the domains of neural markers during gastrulation (Fig. 3). In both wild type and MZ*oep* mutant embryos with compromised FGF signaling, the early neural domain was reduced and shifted closer to the margin. The position of the neural domain suggests that it may be generated in response to signals from the margin.

The analysis of transcript levels using real-time PCR revealed a decrease in expression levels of neural markers and corresponding increases in expression levels of markers of the ventral ectoderm. Since the size of the expression domains of markers and their expression levels is likely to correlate to cell numbers, we conclude that blocking FGF signaling increases the number of ectodermal cells adopting nonneural fates and decreases the number of cells adopting neural fates.

FGF has also been implicated as an important posteriorizing signal. If the sole role of FGF was in posteriorization of the neural plate, our manipulations would have resulted in an increase in the expression of anterior neural markers, without an alteration of the expression of markers of nonneural ectoderm. Previous experiments using XFD in zebrafish have led to the conclusion that following XFD treatment *hoxb1b* expression is sharply reduced while *otx2* expression is expanded toward the margin (Kudoh et al., 2002). We also observed repression of *hoxb1b* expression and a vegetal shift of *otx2* expression but we detected a clear decrease in the size of the *otx2* domain and decreases in *otx2* transcript levels. While we do not fully understand the reason for the differences in results, our expression analysis was done at an earlier stage (mid- gastrula) while the other observations were made near the end of gastrulation and *otx2* levels may have begun to recover.

In our experiments, both XFD and Δ-FGFR-4 (data not shown) produced defects in anterior neural tissues, and we did not observe significant differences between these reagents. Since the specificity of these dominant-negative constructs is unknown, we cannot be sure which FGF receptors are being blocked. However, it is plausible that both FGFR-1 and FGFR-4 play a role in mediating neural induction by FGF.

Recovery of neural tissue in embryos with impaired FGF signaling

Our results demonstrate that XFD blocks neural induction at mid-gastrula but that *otx2* expression recovers to wild-type levels by the six-somite stage (Fig. 4). This recovery depends on the mesoderm (Nodal signaling) as *otx2* expression remains reduced in XFD-injected MZ*oep* mutants at the six-somite stage and deficits in anterior neural tissue are apparent in these embryos at 24 h (Fig. 3D). Despite the clear deficits in neural marker expression during gastrulation following inhibition of FGF signaling in wild-type embryos, neural tissue recovers to produce brains of overtly normal size and structure.

These experiments suggest that the ectoderm may remain competent to respond to neural-inducing signals throughout gastrulation. Surprisingly, even though neural marker expression is delayed in the dorsal ectoderm after inhibition of FGF signaling, largely normal morphogenesis and patterning ensue. While we do not know the origin of the *otx2*-expressing cells that we observe at the six-somite stage, they may arise from a late-acting signal that acts on *gata2/3*-positive cells that populate the dorsal ectoderm in embryos with compromised FGF signaling. It is also possible that inductive signals act on a cell population that exists within the ectoderm that expresses neither neural or epidermal markers. Alternatively, the smaller population of anterior neural cells in embryos with impaired FGF signaling may be subject to fewer limitations on proliferation than cells in the wild-type neural domain.

Our results suggest redundant activities between FGF and downstream Nodal target genes in neural induction.

Virtually all mesendodermal genes require Nodal signaling, and the effect of Nodal is likely to be indirect. FGF has been shown to act downstream of Nodal in mesoderm induction in zebrafish embryos (Mathieu et al., 2004), and we cannot rule out the possibility that the Nodal target mediating the recovery is actually a late-acting FGF that was not inhibited by our treatments.

It is also plausible that the recovery of *otx2* expression in MZ*oep* embryos with compromised FGF signaling stems from the abnormal cellular topography created by the failure of mutant marginal cells to involute (Carmany-Rampey and Schier, 2001). Marginal cells in MZ*oep* mutants may remain in contact with the ectoderm for a longer period than wild-type embryos. This additional time may allow inductive interactions that do not occur in wild-type embryos.

Since blocking FGF signaling in *MZoep;dino* double mutants completely abolishes *otx2* expression at mid-gastrulation (Fig. 4H), we conclude that the early neural domain is established by the activities of Chordin, FGF, and Nodal targets acting in concert. The analysis of these mutants also implies that FGF signaling is sufficient to induce some neural markers in the absence of organizer activity and Chordin. This result contrasts with conclusions from the chick that suggest that FGF is not sufficient for neural induction (Streit et al., 2000; Wilson and Rubenstein, 2000). Further experiments will be necessary to determine whether this difference reflects subtleties in the assay conditions or fundamental species-specific mechanisms.

FGF signaling represses BMP activity on multiple levels

Repression of BMP signaling is thought to be a critical step in establishing the neural domain. Our results demonstrate that FGF signaling in the zebrafish blastula is required to suppress expression of BMP ligands. Activation of FGF signaling represses BMP expression (Figs. 6–8), and impeding FGF signaling with XFD, Δ-FGFR-4, or SU5402 (Figs. 5 and 6) results in increased expression levels of *bmp2*, *bmp4*, and *bmp7*. These results extend and support similar recent findings in zebrafish that modulation of FGF activity regulates expression of BMP ligands (Furthauer et al., 2004).

Since the ability of SU5402 to derepress BMP transcript levels diminishes between the 512-cell and sphere stages, the endogenous FGF signaling events must occur around the time of the mid-blastula transition. Therefore, it is likely that the FGF pathway is activated by an early zygotic gene product. Our results are consistent with the findings of Wilson et al. (2000) that demonstrate a role for FGF signaling in repressing BMP transcription in the chick. Together, these data suggest that repression of BMP transcription by FGF signaling may be a common, early step in defining the neural territory in vertebrates.

Transcription of BMPs is also a readout of BMP signaling since BMPs autoregulate their own transcription (Hild et al., 1999; Jones et al., 1992). However, two lines of evidence indicate that the early effect we observe on BMP transcript levels is not due to blocking BMP autoregulation: first, FGF can repress BMP transcript levels in the absence of protein synthesis at early stages (Fig. 8), and second, Chordin overexpression cannot alter levels of BMP transcripts at these stages (Fig. 7M). Therefore, FGF must be acting to repress early zygotic transcription of BMP ligands. In early gastrula embryos, we also observed decreased transcript levels of BMP target genes *vox* and *eve1* in response to upregulated FGF signaling and, conversely, increased levels in response to inhibition of FGF activity (Fig. 6D). This effect could be caused by diminished levels of BMP ligands or by another means of blocking BMP signaling.

We also observed that FGF signaling is required for expression of *chordin* (Fig. 7). However, the finding that BMP transcription can still be repressed in *dino* mutants (which lack Chordin) by activation of FGF signaling (Fig. 7) provides further evidence that FGF must act via additional mechanisms.

Nevertheless, regulation of *chordin* expression by FGF is likely to be an important mechanism by which presumptive neural tissue is protected from the anti-neuralizing effects of BMPs. Additionally, there are other mechanisms of regulation of BMP signaling by FGF signaling. The human Smad1 protein contains four consensus ERK–MAP kinase phosphorylation sites (Kretzschmar et al., 1997). These sites have been shown to negatively regulate Smad1 in response to a receptor tyrosine kinase activity (Kretzschmar et al., 1997; Pera et al., 2003). The role of these sites in blocking BMP autoregulation has not yet been elucidated, although the sites are clearly important in modulating BMP signal transduction.

Responses to FGF signaling during early cell fate choice in the chick are mediated by Smad interacting protein1 (Sip1; Sheng et al., 2003). This mechanism could also be important to the downregulation of BMP transcripts by FGF in zebrafish by suppressing early TGF-β signals that may be required for BMP expression. Indeed, in zebrafish, experimental evidence suggests that some BMP expression may be regulated by a maternal GDF6-related molecule Radar (Sidi et al., 2003). FGF could downregulate BMP expression by acting via Sip1 to block induction of transcription by Radar. It will be important to determine the early mechanisms by which BMP transcription is repressed by FGF.

BMP transcription is increased when protein synthesis is blocked after the MBT in the absence of additional treatments (Fig. 8). This suggests translation of an early zygotic gene is likely required to suppress BMP transcription. Zygotic transcriptional regulators including the *iroquois* homeobox genes and *bozozok* have been shown to repress BMP transcription and might mediate this activity (Fekany-Lee et al., 2000; Gomez-Skarmeta et al., 2001; Leung et al., 2003). Phosphorylation of Irx2 in response to FGF8 has been shown to be involved in

cerebellum formation in the chick (Matsumoto et al., 2004), and it is plausible that early acting *iroquois* genes may also be regulated by FGF signaling.

The effects of FGF on the initial specification of neural tissue may not be limited to regulation of BMP signaling. Bertrand et al. (2003) have elegantly demonstrated that in ascidians, FGF 9/16/20 directly regulates Otx expression via Ets1/2 and GATAa. A BMP-independent function of FGF has also been suggested in the chick (Wilson et al., 2001). Recently, experiments in zebrafish have demonstrated that FGF is involved in specification of presumptive spinal cord in a ventral domain of the embryo where high BMP activity is present (Kudoh et al., 2004). This finding strongly implies that FGF has activities in neural specification that are independent of regulation of BMP signaling. While the Kudoh study focused on posterior neural specification, FGF may also have similar functions in anterior neural specification.

FGF also plays a direct role in mesoderm formation (Kimelman and Kirschner, 1987), in addition to its roles in neural induction. How these seemingly distinct activities are regulated is an important question. Some insight comes from recent work in the chick that implicates the zinc finger protein Churchill as a switch that modulates the activity of FGF on the mesoderm (Sheng et al., 2003). It has not yet been determined if Churchill has a similar function in zebrafish.

Our results and the work of others suggest a model where FGF acts at multiple levels to suppress BMP signaling. FGF is likely to have additional direct effects on neural specification. Nodal also regulates *chordin* expression and through induction of the mesoderm has additional influences on the neural domain. The clearing of BMP transcripts from the dorsal ectoderm during late blastula stages is mediated by FGF. The endogenous FGF ligand that functions in this context is unknown as is the means by which its activity is regulated. These results support a unifying mechanism for neural induction in vertebrates where FGF acts during the late blastula period to establish a presumptive neural precursor population that is devoid of BMP transcripts. Later signals from the organizer and Chordin serve to protect those precursors from the effects of BMP signaling and to reinforce the neural character of the dorsal ectoderm.

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References

Amaya, E., Musci, T.J., Kirschner, M.W., 1991. Expression of a dominant-negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. Cell 66, 257–270.

Ang, S.L., Rossant, J., 1994. HNF-3 beta is essential for node and notochord formation in mouse development. Cell 78, 561–574.

Bachiller, D., Klingensmith, J., Kemp, C., Belo, J.A., Anderson, R.M., May, S.R., McMahon, J.A., McMahon, A.P., Harland, R.M., Rossant, J., De Robertis, E.M., 2000. The organizer factors Chordin and Noggin are required for mouse forebrain development. Nature 403, 658–661.

Bertrand, V., Hudson, C., Caillol, D., Popovici, C., Lemaire, P., 2003. Neural tissue in ascidian embryos is induced by FGF9/16/20, acting via a combination of maternal GATA and Ets transcription factors. Cell 115, 615–627.

Carmany-Rampey, A., Schier, A.F., 2001. Single-cell internalization during zebrafish gastrulation. Curr. Biol. 11, 1261–1265.

Detrich III, H.W., Kieran, M.W., Chan, F.Y., Barone, L.M., Yee, K., Rundstadler, J.A., Pratt, S., Ransom, D., Zon, L.I., 1995. Intra-embryonic hematopoietic cell migration during vertebrate development. Proc. Natl. Acad. Sci. U. S. A. 92, 10713–10717.

Fekany-Lee, K., Gonzalez, E., Miller-Bertoglio, V., Solnica-Krezel, L., 2000. The homeobox gene bozozok promotes anterior neuroectoderm formation in zebrafish through negative regulation of BMP2/4 and Wnt pathways. Dev. Suppl. 127, 2333–2345.

Feldman, B., Gates, M.A., Egan, E.S., Dougan, S.T., Rennebeck, G., Sirotkin, H.I., Schier, A.F., Talbot, W.S., 1998. Zebrafish organizer development and germ-layer formation require nodal-related signals. Nature 395, 181–185.

Feldman, B., Dougan, S.T., Schier, A.F., Talbot, W.S., 2000. Nodal-related signals establish mesendodermal fate and trunk neural identity in zebrafish. Curr. Biol. 10, 531–534.

Furthauer, M., Van Celst, J., Thisse, C., Thisse, B., 2004. Fgf signalling controls the dorsoventral patterning of the zebrafish embryo. Development 131, 2853–2864.

Gomez-Skarmeta, J., de La Calle-Mustienes, E., Modolell, J., 2001. The Wnt-activated Xiro1 gene encodes a repressor that is essential for neural development and downregulates Bmp4. Development 128, 551–560.

Griffin, K., Patient, R., Holder, N., 1995. Analysis of FGF function in normal and no tail zebrafish embryos reveals separate mechanisms for formation of the trunk and the tail. Dev. Suppl. 121, 2983–2994.

Griffin, K.J., Amacher, S.L., Kimmel, C.B., Kimelman, D., 1998. Molecular identification of spadetail: regulation of zebrafish trunk and tail mesoderm formation by T-box genes. Dev. Suppl. 125, 3379–3388.

Grinblat, Y., Sive, H., 2001. zic gene expression marks anteroposterior pattern in the presumptive neurectoderm of the zebrafish gastrula. Dev. Dyn. 222, 688–693.

Grinblat, Y., Gamse, J., Patel, M., Sive, H., 1998. Determination of the zebrafish forebrain: induction and patterning. Development 125, 4403–4416.

Gritsman, K., Zhang, J., Cheng, S., Heckscher, E., Talbot, W.S., Schier, A.F., 1999. The EGF–CFC protein one-eyed pinhead is essential for nodal signaling. Cell 97, 121–132.

Halpern, M.E., Ho, R.K., Walker, C., Kimmel, C.B., 1993. Induction of muscle pioneers and floor plate is distinguished by the zebrafish no tail mutation. Cell 75, 99–111.

Hammerschmidt, M., Pelegri, F., Mullins, M.C., Kane, D.A., van Eeden, F.J., Granato, M., Brand, M., Furutani-Seiki, M., Haffter, P., Heisenberg, C.P., et al., 1996. Dino and Mercedes, two genes regulating dorsal development in the zebrafish embryo. Dev. Suppl. 123, 95–102.

Hemmati-Brivanlou, A., Melton, D., 1997. Vertebrate neural induction. Annu. Rev. Neurosci. 20, 43–60.

Hild, M., Dick, A., Rauch, G.J., Meier, A., Bouwmeester, T., Haffter, P.,

Hammerschmidt, M., 1999. The smad5 mutation somitabun blocks Bmp2b signaling during early dorsoventral patterning of the zebrafish embryo. Development 126, 2149–2159.

Hongo, I., Kengaku, M., Okamoto, H., 1999. FGF signaling and the anterior neural induction in *Xenopus*. Dev. Biol. 216, 561–581.

Hug, B., Walter, V., Grunwald, D.J., 1997. tbx6, a Brachyury-related gene expressed by ventral mesendodermal precursors in the zebrafish embryo. Dev. Biol. 183, 61–73.

Joly, J.S., Joly, C., Schulte-Merker, S., Boulekbache, H., Condamine, H., 1993. The ventral and posterior expression of the zebrafish homeobox gene eve1 is perturbed in dorsalized and mutant embryos. Development 119, 1261–1275.

Jones, C.M., Lyons, K.M., Lapan, P.M., Wright, C.V., Hogan, B.L., 1992. DVR-4 (bone morphogenetic protein-4) as a posterior-ventralizing factor in *Xenopus* mesoderm induction. Development 115, 639–647.

Kiefer, P., Strahle, U., Dickson, C., 1996. The zebrafish Fgf-3 gene: cDNA sequence, transcript structure and genomic organization. Gene 168, 211–215.

Kimelman, D., Kirschner, M., 1987. Synergistic induction of mesoderm by FGF and TGF-beta and the identification of an mRNA coding for FGF in the early *Xenopus* embryo. Cell 51, 869–877.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310.

Kishimoto, Y., Lee, K.H., Zon, L., Hammerschmidt, M., Schulte-Merker, S., 1997. The molecular nature of zebrafish swirl: BMP2 function is essential during early dorsoventral patterning. Development 124, 4457–4466.

Klingensmith, J., Ang, S.L., Bachiller, D., Rossant, J., 1999. Neural induction and patterning in the mouse in the absence of the node and its derivatives. Dev. Biol. 216, 535–549.

Koshida, S., Shinya, M., Nikaido, M., Ueno, N., Schulte-Merker, S., Kuroiwa, A., Takeda, H., 2002. Inhibition of BMP activity by the FGF signal promotes posterior neural development in zebrafish. Dev. Biol. 244, 9–20.

Krauss, S., Johansen, T., Korzh, V., Fjose, A., 1991. Expression of the zebrafish paired box gene pax[zf-b] during early neurogenesis. Development 113, 1193–1206.

Kretzschmar, M., Doody, J., Massague, J., 1997. Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. Nature 389, 618–622.

Kroll, K.L., Amaya, E., 1996. Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. Development 122, 3173–3183.

Kudoh, T., Wilson, S.W., Dawid, I.B., 2002. Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm. Development 129, 4335–4346.

Kudoh, T., Concha, M.L., Houart, C., Dawid, I.B., Wilson, S.W., 2004. Combinatorial Fgf and Bmp signalling patterns the gastrula ectoderm into prospective neural and epidermal domains. Development 131, 3581–3592.

Kuroda, H., Wessely, O., Robertis, E.M., 2004. Neural induction in *Xenopus*: requirement for ectodermal and endomesodermal signals via chordin, noggin, beta-catenin, and cerberus. PLoS Biol. 2, E92.

Leung, T., Bischof, J., Soll, I., Niessing, D., Zhang, D., Ma, J., Jackle, H., Driever, W., 2003. Bozozok directly represses bmp2b transcription and mediates the earliest dorsoventral asymmetry of bmp2b expression in zebrafish. Development 130, 3639–3649.

Mathieu, J., Griffin, K., Herbomel, P., Dickmeis, T., Strahle, U., Kimelman, D., Rosa, F.M., Peyrieras, N., 2004. Nodal and Fgf pathways interact through a positive regulatory loop and synergize to maintain mesodermal cell populations. Development 131, 629–641.

Matsumoto, K., Nishihara, S., Kamimura, M., Shiraishi, T., Otoguro, T., Uehara, M., Maeda, Y., Ogura, K., Lumsden, A., Ogura, T., 2004. The prepattern transcription factor Irx2, a target of the FGF8/MAP kinase cascade, is involved in cerebellum formation. Nat. Neurosci.

Melby, A.E., Beach, C., Mullins, M., Kimelman, D., 2000. Patterning the early zebrafish by the opposing actions of bozozok and vox/vent. Dev. Biol. 224, 275–285.

Miller-Bertoglio, V.E., Fisher, S., Sanchez, A., Mullins, M.C., Halpern, M.E., 1997. Differential regulation of chordin expression domains in mutant zebrafish. Dev. Biol. 192, 537–550.

Mohammadi, M., Schlessinger, J., Hubbard, S.R., 1996. Structure of the FGF receptor tyrosine kinase domain reveals a novel autoinhibitory mechanism. Cell 86, 577–587.

Mori, H., Miyazaki, Y., Morita, T., Nitta, H., Mishina, M., 1994. Different spatio-temporal expressions of three otx homeoprotein transcripts during zebrafish embryogenesis. Brain Res. Mol. Brain Res. 27, 221–231.

Morita, T., Nitta, H., Kiyama, Y., Mori, H., Mishina, M., 1995. Differential expression of two zebrafish emx homeoprotein mRNAs in the developing brain. Neurosci. Lett. 198, 131–134.

Munoz-Sanjuan, I., Brivanlou, A.H., 2002. Neural induction, the default model and embryonic stem cells. Nat. Rev., Neurosci. 3, 271–280.

Nasevicius, A., Ekker, S.C., 2000. Effective targeted gene 'knockdown' in zebrafish. Nat. Genet. 26, 216–220.

Neave, B., Rodaway, A., Wilson, S.W., Patient, R., Holder, N., 1995. Expression of zebrafish GATA 3 (gta3) during gastrulation and neurulation suggests a role in the specification of cell fate. Mech. Dev. 51, 169–182.

Nieukoop, P., 1952. Activation and organization of the central nervous system in amphibians. J. Exp. Zool. 120, 1–108.

Nikaido, M., Tada, M., Saji, T., Ueno, N., 1997. Conservation of BMP signaling in zebrafish mesoderm patterning. Mech. Dev. 61, 75–88.

Oelgeschlager, M., Kuroda, H., Reversade, B., De Robertis, E.M., 2003. Chordin is required for the spemann organizer transplantation phenomenon in *Xenopus* embryos. Dev. Cell 4, 219–230.

Oxtoby, E., Jowett, T., 1993. Cloning of the zebrafish krox-20 gene (krx-20) and its expression during hindbrain development. Nucleic Acids Res. 21, 1087–1095.

Pera, E.M., Ikeda, A., Eivers, E., De Robertis, E.M., 2003. Integration of IGF, FGF, and anti-BMP signals via Smad1 phosphorylation in neural induction. Genes Dev. 17, 3023–3028.

Piccolo, S., Sasai, Y., Lu, B., De Robertis, E.M., 1996. Dorsoventral patterning in *Xenopus*: inhibition of ventral signals by direct binding of chordin to BMP-4. Cell 86, 589–598.

Pownall, M.E., Welm, B.E., Freeman, K.W., Spencer, D.M., Rosen, J.M., Isaacs, H.V., 2003. An inducible system for the study of FGF signalling in early amphibian development. Dev. Biol. 256, 89–99.

Ribisi Jr., S., Mariani, F.V., Aamar, E., Lamb, T.M., Frank, D., Harland, D.M., 2000. Ras-mediated FGF signaling is required for the formation of posterior but not anterior neural tissue in *Xenopus laevis*. Dev. Biol. 227, 183–196.

Sasai, Y., Lu, B., Steinbelsser, H., De Robertis, E.M., 1995. Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in *Xenopus*. Nature 378, 419.

Saude, L., Woolley, K., Martin, P., Driever, W., Stemple, D.L., 2000. Axis-inducing activities and cell fates of the zebrafish organizer. Development 127, 3407–3417.

Schmid, B., Furthauer, M., Connors, S.A., Trout, J., Thisse, B., Thisse, C., Mullins, M.C., 2000. Equivalent genetic roles for bmp7/snailhouse and bmp2b/swirl in dorsoventral pattern formation. Dev., Suppl. 127, 957–967.

Schulte-Merker, S., Lee, K.J., McMahon, A.P., Hammerschmidt, M., 1997. The zebrafish organizer requires chordino. Nature 387, 862–863.

Schulte-Merker, S., van Eeden, F.J., Halpern, M.E., Kimmel, C.B., Nusslein-Volhard, C., 1994. No tail (ntl) is the zebrafish homologue of the mouse T (Brachyury) gene. Development 120, 1009–1015.

Sheng, G., dos Reis, M., Stern, C.D., 2003. Churchill, a zinc finger transcriptional activator, regulates the transition between gastrulation and neurulation. Cell 115, 603–613.

Sidi, S., Goutel, C., Peyrieras, N., Rosa, F.M., 2003. Maternal induction of ventral fate by zebrafish radar. Proc. Natl. Acad. Sci. U. S. A. 100, 3315–3320.

Sirotkin, H.I., Dougan, S.T., Schier, A.F., Talbot, W.S., 2000. Bozozok and

squint act in parallel to specify dorsal mesoderm and anterior neuroectoderm in zebrafish. Dev., Suppl. 127, 2583–2592.
Smith, W.C., Harland, R.M., 1992. Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in *Xenopus* embryos. Cell 70, 829–840.
Spemann, H.a.M.H., 1924. Ueber die Induktion von Embryoanalangen durch Inplantation artfremder Organisatoren. Wilhelm Roux' Arch. Entwickl. Mech. 100, 599–638.
Stern, C.D., 2002. Induction and initial patterning of the nervous system—The chick embryo enters the scene. Curr. Opin. Genet. Dev. 12, 447–451.
Stern, C.D., Jaques, K.F., Lim, T.M., Fraser, S.E., Keynes, R.J., 1991. Segmental lineage restrictions in the chick embryo spinal cord depend on the adjacent somites. Dev., Suppl. 113, 239–244.
Streit, A., Lee, K.J., Woo, I., Roberts, C., Jessell, T.M., Stern, C.D., 1998. Chordin regulates primitive streak development and the stability of induced neural cells, but is not sufficient for neural induction in the chick embryo. Development 125, 507–519.
Streit, A., Berliner, A.J., Papanayotou, C., Sirulnik, A., Stern, C.D., 2000. Initiation of neural induction by FGF signalling before gastrulation. Nature 406, 74–78.
Thisse, C., Thisse, B., Schilling, T.F., Postlethwait, J.H., 1993. Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. Development 119, 1203–1215.
Welm, B.E., Freeman, K.W., Chen, M., Contreras, A., Spencer, D.M., Rosen, J.M., 2002. Inducible dimerization of FGFR1: development of a mouse model to analyze progressive transformation of the mammary gland. J. Cell Biol. 157, 703–714.
Wilson, S.W., Rubenstein, J.L., 2000. Induction and dorsoventral patterning of the telencephalon. Neuron 28, 641–651.
Wilson, S.I., Graziano, E., Harland, R., Jessell, T.M., Edlund, T., 2000. An early requirement for FGF signalling in the acquisition of neural cell fate in the chick embryo. Curr. Biol. 10, 421–429.
Wilson, S.I., Rydstrom, A., Trimborn, T., Willert, K., Nusse, R., Jessell, T.M., Edlund, T., 2001. The status of Wnt signalling regulates neural and epidermal fates in the chick embryo. Nature 411, 325–330.
Woo, K., Fraser, S.E., 1997. Specification of the zebrafish nervous system by nonaxial signals. Science 277, 254–257.
Yamamoto, A., Amacher, S.L., Kim, S.H., Geissert, D., Kimmel, C.B., De Robertis, E.M., 1998. Zebrafish paraxial protocadherin is a downstream target of spadetail involved in morphogenesis of gastrula mesoderm. Development 125, 3389–3397.
Zimmerman, L.B., De Jesus-Escobar, J.M., Harland, R.M., 1996. The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. Cell 86, 599–606.